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14. ABSTRACT
The project produced initial characterization of xanthorhodopsin, a novel carotenoid/retinal protein complex from the extremely halophilic eubacterium Salinibacter ruber, which functions as a light-driven proton pump. Its unique feature is a light-harvesting antenna, not found in any other retinal-based pumps. Fluorescence studies showed that 45% of light quanta absorbed by the carotenoid salinixanthin is transferred to retinal and used for transmembrane proton transport to energize ATP synthesis in the cells. The energy transfer occurs from extremely short lived (64

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Report Title

Structure and function of xanthorhodopsin

ABSTRACT

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List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

With the support of the ARO Grant W911NF-06-1-0020 (starting date December 1, 2005, end date February 28, 2009) the following papers have been published in peer reviewed journals by the UCI team:

1. Balashov, S. P., E. S. Imasheva and J. K. Lanyi. 2006. Induced chirality of light-harvesting caro-tenoid salinixanthin and its interaction with the retinal of xanthorhodopsin. Biochemistry 45, 10998-11004.

2. Imasheva, E. S., S. P. Balashov, J. M. Wang and J. K. Lanyi. 2006. pH-dependent transitions in xanthorhodopsin. Photochem. Photobiol. 82, 1406-1413.

3. Boichenko, V. A., J. M. Wang, J. Antón, J. K. Lanyi and S. P. Balashov. 2006. Functions of ca-rotenoids in xanthorhodopsin and archaerhodopsin, from action spectra of photoinhibition of cell respiration. Biochim. Biophys. Acta 1757, 1649-1656.

4. Balashov, S.P. and J. K. Lanyi. 2007. Xanthorhodopsin: proton pump with a carotenoid antenna. Cell. Mol. Life Sci. 64 (2007) 2323-2328.

5. Imasheva, E. S., S. P. Balashov, J. M. Wang, E. Smolensky, M. Sheves and J. K. Lanyi. 2008. Chromophore interaction in xanthorhodopsin – retinal dependence of salinixanthin binding. Pho-tochem. Photobiol. 84, 977-984.

6. Lanyi, J. K., S.P. Balashov. 2008. Xanthorhodopsin: a bacteriorhodopsin-like proton pump with a carotenoid antenna. Biochimica et Biophysica Acta 1777, 684-688.

7. Balashov, S. P., E. S. Imasheva, J. M. Wang, and J. K. Lanyi. 2008. Excitation energy-transfer and the relative orientation of retinal and carotenoid in xanthorhodopsin. Biophys. J. 95: 2402-2414.

8. Luecke, H., B. Schobert, J. Stagno, E. S. Imasheva, J. M. Wang, S. P. Balashov and J. K. Lanyi. 2008. Crystal structure of the dual-chromophore light-driven proton pump xanthorhodopsin. Proc. Natl. Acad. Sci. USA 105: 16561-16565.

9. Polívka, T., S. P. Balashov, P. Chábera, E. S. Imasheva, A. Yartsev, V. Sundström and J. K. Lanyi. Femtosecond Carotenoid to Retinal Energy Transfer in Xanthorhodopsin. 2009. Biophys. J. 96, 2268-2277.

Number of Papers published in peer-reviewed journals: 9.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals: 0.00

(c) Presentations

The results were presented at 11 Conferences and Meetings and, though not published in Conference Proceedings, have been published as regular papers.

Number of Presentations: 11.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Number of Manuscripts: 0.00

Number of Inventions:

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Eleonora Imasheva	0.20
FTE Equivalent:	**0.20**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Janos Lanyi	0.05	No
Sergei Balashov	0.20	No
FTE Equivalent:	**0.25**	
Total Number:	**2**	

Names of Under Graduate students supported

NAME	PERCENT SUPPORTED
Linda Uy (in 2007)	0.15
Daniel Oh (in 2008)	0.20
FTE Equivalent:	**0.35**
Total Number:	**2**

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
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Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT SUPPORTED	
Jennifer Wang	0.20	No
FTE Equivalent:	**0.20**	
Total Number:	**1**	

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STRUCTURE AND FUNCTION OF XANTHORHODOPSIN

Final report on the ARO GRANT W911NF-06-1-0020
"Study of Xanthorhodopsin, the Retinal-Protein Proton Pump of *Salinibacter ruber* with Light-Harvesting Carotenoid Antenna". March 19, 2009.

S. P. Balashov, PI and J. K. Lanyi, Co-PI

Dept. of Physiology and Biophysics, University of California, Irvine

CONTENT

Abstract. The project produced initial characterization of xanthorhodopsin, a novel carotenoid/retinal protein complex from the extremely halophilic eubacterium *Salinibacter ruber*, which functions as a light-driven proton pump. Its unique feature is a light-harvesting antenna, not found in any other retinal-based pumps. Fluorescence studies showed that 45% of light quanta absorbed by the carotenoid salinixanthin is transferred to retinal and used for transmembrane proton transport to energize ATP synthesis in the cells. The energy transfer occurs from extremely short lived (64 femtosecond) excited state of the carotenoid S2 to the S1 state of the retinal. From spectroscopic and x-ray studies that produced a 1.9 Angstom resolution structure, important information was obtained on the location of the carotenoid in the protein and its interaction with the retinal. The structure also revealed a number of unexpected features in the arrangement of the proton conducting pathways, not seen in the archaeal pumps, bacteriorhodopsin and archaerhodopsin. Particularly significant is a strongly hydrogen bonded Asp-His pair as the counterion to the retinal Schiff base, and a large cleft that extends deep into the protein at the extracellular side. It is likely that these features are common to other bacterial pumps, the proteorhodopsins, which still lack crystallographic information.

Introduction. This report describes the results of the study of xanthorhodopsin under the ARO Grant (December 2005 – February 2009), which began soon after the discovery of this retinal protein-based proton pump with a carotenoid light-harvesting antenna in extremely halophilic eubacterium *Salinibacter ruber* [1]. As any groundbreaking discovery, it raised a number of questions. They were about the "architecture" of this unusual proton pump, and the way the large carotenoid salinixanthin (**Figure 1**) is accommodated by the relatively small protein, as well as about the distinctive features of xanthorhodopsin in comparison with earlier studied proton pumps with retinal chromophore but without an antenna (bacteriorhodopsin and archaerhodopsin of halobacteria, and proteorhodopsin of marine bacteria).

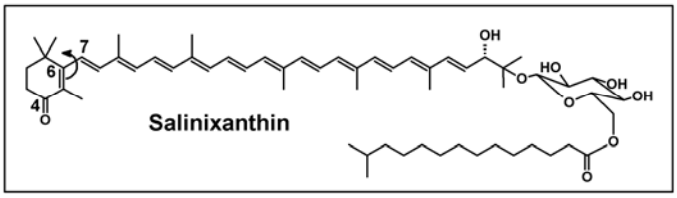

Figure 1. Chemical structure of carotenoid salinixanthin from the cell membrane of *Salinibacter ruber.* After Lutnaes et al. [2]. An arrow around C6-C7 bond shows a turn of the ring in xanthorhodopsin [3, 4].

As of the beginning of 2009, all available publications on xanthorhodopsin [1, 3-11] were from our group, except the Mongodin et al. paper [12] which described the genome of *Salinibacter ruber* and the relationship of the xanthorhodopsin gene to other retinal proteins (**Figure 2**).

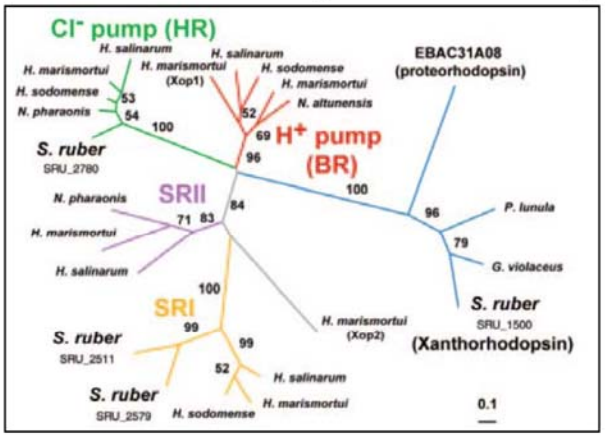

Figure 2. A likelihood phylogeny of retinal protein genes of *S. ruber*. Note the distant relationship of xanthorhodopsin to the archaeal proton pump bacteriorhodopsin, and the much greater homology to rhodopsins of *Gloeobacter violaceus*. Three other genes of *S. ruber* coding halorhodopsin (HR) and two sensory rhodopsin-I like proteins (SRI) are close to archaeal homologs. From [12].

These findings have sparked the interest in xanthorhodopsin, and several groups (in USA, Spain, Sweden, Czech Republic, Israel, Japan, Germany and Russia) have already entered the field through collaboration with us or independently. Recently, a xanthorhodopsin-like gene was found in the genome of an abundant coastal ocean methylotroph that utilizes methanol and formaldehyde as sources of carbon [13]. This gene forms a clade with the xanthorhodopsin of *Salinibacter ruber* and rhodopsins of *Gloeobacter violaceus* and several other divergent organisms. The finding indicates that the xanthorhodopsin-like retinal proteins might be as widespread as the homologous proteorhodopsins [14, 15]. Below, we summarize our studies of xanthorhodopsin, which culminated in its crystallization and solution of its structure to 1.9 Å resolution.
All major directions that were proposed to explore under the ARO Grant have been explored and the results met or surpassed our expectations. In the following we describe the main findings.

Results and conclusions

1. Spectroscopic evidence for a specific binding site of the carotenoid antenna; chirality of salinixanthin in xa**nthorhodopsin [3].** Evidence for a specific and rigid binding of the carotenoid antenna in xanthorhodopsin was obtained in experiments in which the retinal chromophore was removed and then reconstituted with all-*trans* retinal [1, 3]. Incubation with hydroxylamine results in cleavage of the C=N bond connecting retinal to the protein and elimination of the 560 nm retinal chromophore band which shifts to 368 nm upon formation of retinal oxime (**Figure 3**). This is typical for retinal proteins. Surprisingly, the carotenoid bands were also strongly affected (curve 2 in Figure 3). They became less intense and broader compared to that in xanthorhodopsin spectrum (curve 1) where they are present as well resolved peaks at 521, 486 and 560 nm. Addition of retinal to a protein treated with hydroxylamine resulted in reformation of the C=N bond and reappearance of retinal protein band, accompanied by large changes in the absorption spectrum of the carotenoid: the vibronic bands became much narrower and more intense as in initial pigment (curve 1), apparently from reduction of conformational heterogeneity of the carotenoid by restricting angular movement of its ring around the C6-C7 bond [3]. These changes are induced by insertion of the retinal into its binding site and occur even before (and in the absence of) formation of the protonated Schiff base [9]. This implies that there is interaction between the rings of the two chromophores, recently confirmed in x-ray crystal structure [4], see below.

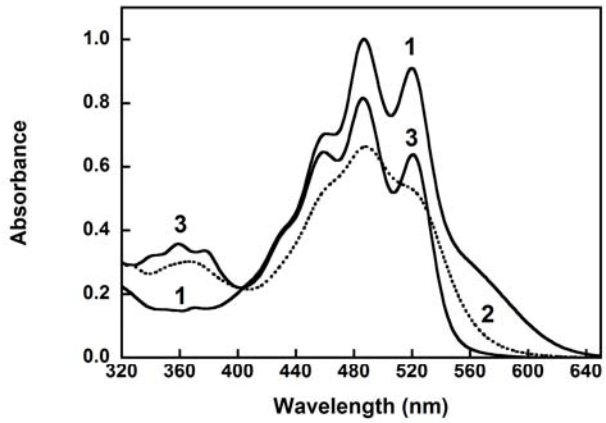

Figure 3. Absorption spectra of: 1, fraction of cell membranes containing xanthorhodopsin; 2, after incubation with 0.2 M hydroxylamine during illumination (to hydrolyze the C=N bond and remove retinal from the binding site); 3, after incubation with sodium borohydride to reduce the C=N bond of the Schiff base to a single bond C-N. The latter treatment keeps retinal attached in its binding site and does not perturb the carotenoid binding site as follows from sharp carotenoid bands [10, 11].

In the unbound state, salinixanthin does not exhibit optical activity, but in the native xanthorhodopsin salinixanthin becomes chiral from an asymmetric conformation, as detected by circular dichroism (CD) spectra [3]. The carotenoid conformation is controlled by the retinal. Hydrolysis of the retinal Schiff base with hydroxylamine and removal of retinal from the binding site eliminates the optical activity of salinixanthin. This shows that in the native state, the protein and the retinal forces its antenna into an asymmetric conformation involving a turn of its ring [3]. Analogous experiments with archaerhodopsin which contains carotenoid bacterioruberin indicated far less coupling of bacterioruberin with retinal, consistent with the observed lack of energy transfer between the two chromophores in this protein [6], see below.

2. Action spectra of xanthorhodopsin in live *Salinibacter ruber* cells; comparison with spectra of archaerhodopsin in *Halorubrum species* [6]. Illumination of *Salinibacter ruber* cells causes decrease of respiration (detected as a decrease in oxygen consumption) due to backpressure of the light-induced electrochemical potential created by xanthorhodopsin on the electron transfer steps in the respiratory chain [6]. An action spectrum for photoinhibition of respiration in *Salinibacter ruber* cells was obtained with high spectral resolution (4 nm). It confirmed our earlier conclusion, based on a spectrum with a lower resolution [1], about the participation of the carotenoid in light-harvesting for proton pumping. The high accuracy of the spectrum enabled us to deconvolute it into two components: retinal and carotenoid **(Figure 4).** The derived spectrum of the carotenoid component is particularly useful since the cell membranes always contain an unknown fraction of carotenoid unbound to xanthorhodopsin, which complicates determination of the exact spectrum of the bound component when using absorption spectroscopy.

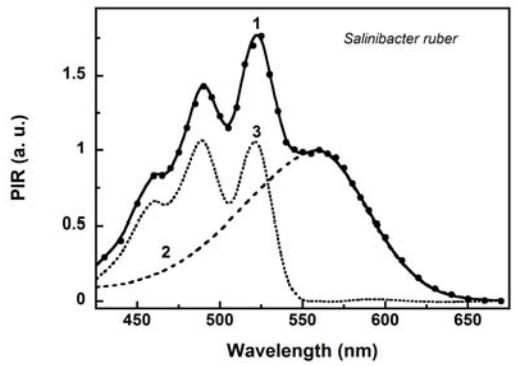

Figure 4. Action spectrum (1) of photoinhibition of respiration (PIR) in *Salinibacter ruber* cells and its retinal (2) and carotenoid (3) components. It shows that light absorbed by carotenoid decreases the rate of cell respiration (as a result of back pressure of light-induced electrochemical proton gradient). From [6].

Action spectra measurements in the cells of the archaeon *Halorubrum* sp. A1C, isolated by our group, and containing archaerhodopsin and the carotenoid bacterioruberin, showed that, unlike in xanthorhodopsin, there is no energy transfer from bacterioruberin to archaerhodopsin [6]. This emphasizes the uniqueness of xanthorhodopsin and implies that antenna function utilizing excitation energy transfer did not emerge among the archaea but appeared later during evolution. The function of bacterioruberin in archaeal membranes is primarily photoprotection and structural stability, whereas salinixanthin has a clear energetic role, perhaps in addition to the two others.

3. The pK_a of the counterion and pH dependence of the photocycle of xanthorhodopsin [7]. The pK_a (proton affinity) of the counterion to the Schiff base in xanthorhodopsin was determined to be 6-6.5 (lower value is in detergent), closer to that in proteorhodopsin (7.5) than to bacterior-

hodopsin (2.6) or archaerhodopsin (3.5). In the latter microbial retinal proteins the pK_a of the counterion can be easily determined by a large (30-40 nm) red shift of retinal absorption spectrum at low pH. Surprisingly, in xanthorhodopsin only a small (3 nm) red shift between pH 2 and 12, with a pK_a 6 takes place. An unusually small shift indicated different structure of the counterion. In order to prove that this shift indeed comes from the protonation of the counterion, the pH dependence of the yield of the M intermediate was determined, which is another indication of the counterion protonation because the counterion serves as proton acceptor during the photocycle. The yield of M decreases with a pK_a of 6.0 with decreasing the pH. This provided independent evidence that the pK_a of the counterion in xanthorhodopsin is 6.0 [7]. This places it as a pump closer to proteorhodopsin than to bacteriorhodopsin. Protonation of the counterion caused only a very small (0.5 nm blue shift) in the spectrum of the carotenoid antenna.

Studies of the pH dependence of the recovery of the initial state in the photocycle of xanthorhodopsin yielded two pK_a's, 6.0 and 9.3. The former is the pK_a of the counterion. The latter has been attributed to the pK_a of the internal proton donor to the retinal Schiff base [7], which is 2 pH units higher than in bacteriorhodopsin [16].

Illumination of xanthorhodopsin at low pH results in formation of long-lived photoproducts. Remarkably, the antenna carotenoid is greatly affected in these states [7], indicating a connection between the isomeric state of the retinal chromophore and the carotenoid.

4. Fluorescence of the retinal chromophore: estimation of efficiency of energy transfer from the excitation spectra [11]. Initially, the evidence for energy transfer from the carotenoid to retinal was obtained by measuring action spectra for proton transport and photoinhibition of respiration in *Salinibacter ruber* cells [1, 6]. It was desirable to obtain independent evidence for the transfer at the very first step of the process, population of the retinal excited state, by detecting fluorescence of the retinal chromophore induced by quanta absorbed by the carotenoid. That would eliminate alternative interpretations of the action spectra, namely that the carotenoid is engaged in proton transfer through unknown mechanism, or in some manner regulates the functioning of retinal but does not supply the energy for it. This was accomplished by detecting fluorescence of the retinal from its strongly allowed $1B_u^+$ excited state (using the C_{2h} symmetry group notation) which in retinal proteins with protonated Schiff bases is the lowest excited state, S_1 state, and measuring its excitation spectrum. The presence of the carotenoid bands in the excitation spectrum provided the final evidence for the transfer [10, 11].

This task was challenging because the retinal fluorescence is weak (quantum yield ca. 2 to 5 x 10^{-4}, depending on pH), so the sample should be free from other fluorescing impurities and exhibit low light-scattering. The instrument equipped with polarizers to allow measurements under "magic angle" conditions enabled us to correctly account for the emission from all molecules regardless of their orientation in the sample. Under these conditions we obtained the fluorescence spectrum of the retinal chromophore (maximum at 690 nm) and the excitation spectrum for the retinal emission [11], shown in **Figure 5**.

The excitation spectrum for the retinal emission sampled at 720 nm (curve 3) is very similar to the excitation spectrum that was obtained earlier for the physiological responses, i.e., light-induced proton transfer and photoinhibition of respiration [1, 6]. From the comparison of

the relative amplitude of the carotenoid bands in the excitation and absorption spectra, we estimated the efficiency of energy transfer from salinixanthin to retinal as 45 ± 5% [11].

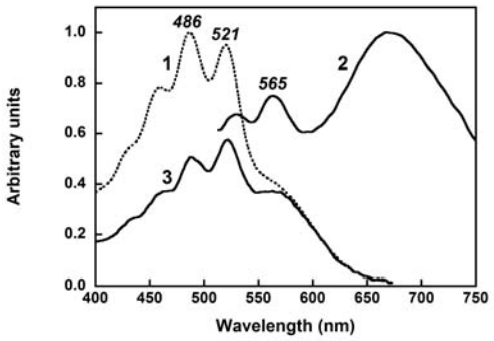

Figure 5. The absorption (1), fluorescence (2) and fluorescence excitation (3) spectra of cell membranes containing xanthorhodopsin. The fluorescence spectrum was obtained upon excitation at 470 nm. The bands at 529 and 565 nm are from salinixanthin emission. A broader band with maximum at 690 nm belongs to retinal chromophores fluorescence. From [10].

5. Detection of the weak fluorescence from the S_2 excited state of salinixanthin [11]. The contribution of carotenoid emission to the total emission at 720 nm (where the retinal emission was sampled) was small, as the close correspondence of the "physiological" action spectrum and fluorescence excitation spectrum indicated. Still, an estimate of this contribution was necessary, from the fluorescence spectrum of carotenoid. Detection of this emission is important also for understanding the mechanism of energy transfer in xanthorhodopsin. Fluorescence of carotenoids with long conjugated chains has been detected before from solutions of carotenoids in organic solvents. It occurs from a $^1B_u^+$ - like state (S_2), and is extremely weak (reviewed in [17]).

Three weak but sharp bands (compared to the retinal bands and the background signal) at 529, 565 and 595-605 nm in xanthorhodopsin fluorescence spectrum were identified as the carotenoid emission [11]. These bands (curve 2 in **Figure 5**) exhibited the features peculiar to the fluorescence from the S_2 excited states of carotenoids with long conjugated chains studied in organic solvents: very low quantum yield ($4 \cdot 10^{-5}$), a small Stokes shift (ca. 300 cm^{-1}), and an approximate mirror image symmetry of the absorption and fluorescence spectra. The quantum yield corresponds to a lifetime of the excited state of about 70 fs [11]. The possible contribution of this emission to the total emission at 720 nm is less than 5% in xanthorhodopsin.

6. Energy transfer occurs from the S_2 excited state of salinixanthin to S_1 state of the retinal [11]. Intense absorption bands of carotenoids are from transition to the S_2 excited state. Transitions from the ground S_0 state to the S_1 state are forbidden but this state is populated in the process of internal conversion from S_2. In light-harvesting complexes of green plants and photosynthetic bacteria, the carotenoid to chlorophyll energy transfer occurs both from the S_2 and S_1 excited state levels of a carotenoid [17]. In xanthorhodopsin the S_1 level cannot serve as a donor because it is below the S_1 level of the retinal chromophore. The estimate based on results from other carotenoids with long chains places the S_1 level of salinixanthin in the near IR region, between 750 and 850 nm, far below the 560 nm absorption band of retinal [11]. We suggested that energy transfer occurs from the S_2 excited state level of carotenoid to S_1 level of retinal [1].

This prediction was confirmed in experiments with borohydride [11]. Reduction of the double C=N bond of the retinal Schiff base to a single bond with sodium borohydride shifts the absorption of retinal from 560 to 360 nm, with no (or almost no) effect on the carotenoid absorption bands because retinal remains in the binding site (**Figure 4**, curve 3). The large blue-shift of

the retinal band eliminates any possibility of energy transfer to the retinal since its energy level becomes much higher than that of the carotenoid. Under these conditions we observed a two-fold increase in the intensity of the carotenoid emission at 529 and 565 nm, originating from the short lived S_2 level. This provides evidence that the S_2 level is the only (or the major) source of energy for the S_1 state of retinal **(Figure 6)**.

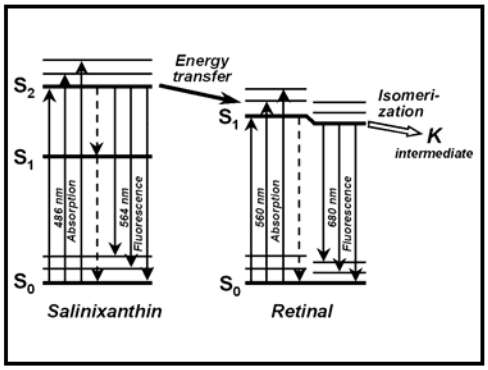

Figure 6. Scheme of excited states of the retinal and carotenoid chromophores of xanthorhodopsin involved in energy transfer pathway. The latter occurs from the same S_2 state, involved in absorption of light by salinixanthin. After [11].

The distance between the centers of the two chromophores was estimated to be ca. 11 Å [11]. The S_2 state is very short-lived (ca. 70 fs), and efficient energy transfer requires close proximity to the acceptor. The efficiency of the excitation energy transfer between donor and acceptor depends on the overlap integral of the donor fluorescence and acceptor absorption. The fluorescence from the S_2 level of carotenoid with maxima at 529 nm and 565 nm optimally overlaps with retinal absorption, with a maximum at 560 nm. Calculations using the Förster equation for the efficiency of energy transfer and the experimentally obtained values for overlap integrals and quantum yield of carotenoid S_2 fluorescence yielded a distance between the centers of the carotenoid polyene chain and the retinal of ca. 11 Å. This is a rough estimate since the dimensions of the chromophores are comparable with the distance between them.

7. Mutual orientation of the chromophores from excitation anisotropy; how to build an efficient antenna [11]. The contribution of the carotenoid to the excitation spectrum of the retinal chromophore fluorescence strongly depends on the polarization of the excitation and emission beams [11]. This means that there is a substantial angle (>> 0°) between the transition moments of the $S_0 \rightarrow S_2$ carotenoid absorption and $S_1 \rightarrow S_0$ retinal fluorescence. From measurement of the excitation anisotropy the angle was determined to be 56 ± 3° [11], close to the 46° angle between the axes of the two conjugated chains determined later by x-ray diffraction [4]. The angle is a compromise between the best efficiency for energy transfer (parallel) and the ability to collect incident light by the dual chromophore system at all angles of polarization (perpendicular). In bacteriorhodopsin the retinal is tilted 21° from the membrane plane. The high degree of homology of residues in the retinal binding sites of the two proteins suggests that the retinal will be oriented similarly in xanthorhodopsin. In this case, the carotenoid will be tilted either 13° or 55° to the membrane normal, and the structure of the protein [4] indicated that the latter is the case.

8. Control of the carotenoid conformation by the retinal ring end: study with retinal analogs and retinol [9]. The 13-desmethyl retinal analog as well as the analog with "locked" C13=C14 bond, formed the protonated Schiff base linkage much more slowly than retinal. This provided the opportunity to observe changes in carotenoid binding at various stages. The characteristic sharpening of the carotenoid bands, indicative of its reduced conformational heterogeneity in the binding site, occurs already when retinal occupies the binding site, even before the covalent bond to Lys via a Schiff base is formed. Reconstitution with retinol, where the Schiff base

cannot form but the carotenoid exhibits spectral changes similar to those when retinal is added, provides further evidence that when retinal enters the binding site it causes restriction of conformational heterogeneity of the carotenoid through steric interaction of their moieties.

9. Crystal structure of xanthorhodopsin to 1.9 Å resolution [4]. Solving the x-ray structure of xanthorhodopsin is an exciting breakthrough, which is important not only for this project but for understanding the broader question of the transport mechanism in microbial light-driven proton pumps. Until now no crystal structure was available for numerous proteorhodopsins, which share many common features with xanthorhodopsin.

The crystal structure [4] enabled us to improve the alignment of XR and PR (**Figure 7**), and revealed a number of new important and unexpected features discussed below.

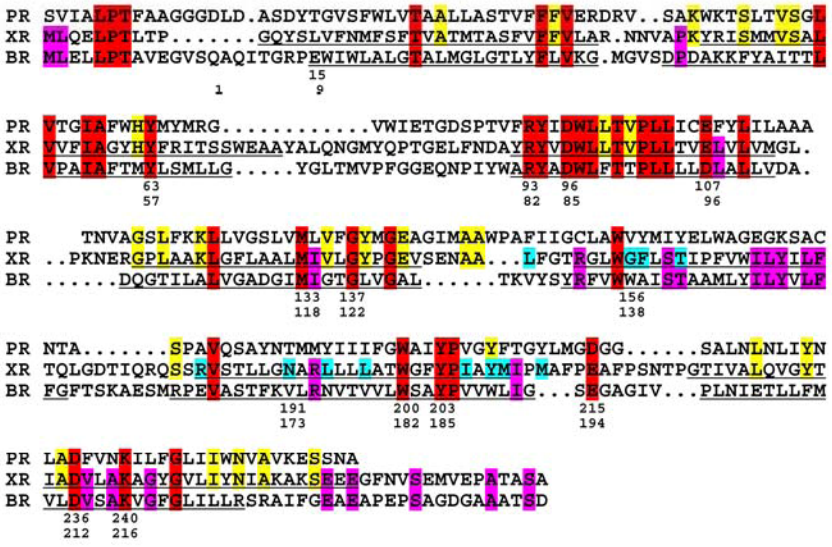

Figure 7. Alignment of amino acid sequences of xanthorhodopsin (XR), proteorhodopsin (PR) and bacteriorhodopsin (BR). Red, conserved in all three proteins; yellow conserved in PR and XR; purple, residues conserved in XR and BR; cyan, residues involved in carotenoid binding. Top row of numbers refer to the XR sequence; bottom row, to the BR sequence. From [4].

a) Relative positions of the two chromophores. The distance between the centers of the two chromophores is 11.7 Å, i.e., about the same as our earlier 11 Å estimate from a simple Förster model for the quenching of carotenoid fluorescence by the retinal [11]. On the other hand, the ring moieties of the chromophores are within only 5 Å of one another, and the retinal β-ionone ring is in van der Waals distance of the carotenoid keto ring (**Figure 8**). Both are in contact with the Tyr^{207} ring between them. This contrasts with the crystal structure of archaerhodopsin [18], a proton pump with a bacterioruberin carotenoid without antenna function, where the corresponding center-to-center distance is 17 Å, with the closest approach of bacterioruberin to the retinal at 12 Å. In that protein the carotenoid may have a structural and photoprotective function. Energy transfer is precluded in archaerhodopsin by a large distance between the chromophores.

The angle between the retinal and salinixanthin axes is 46°, somewhat less than the 56 ± 3° estimated from the polarization anisotropy of retinal fluorescence. The apparent discrepancy most likely originates from the off-axis orientation of the transition moment, as in rhodopin [19].

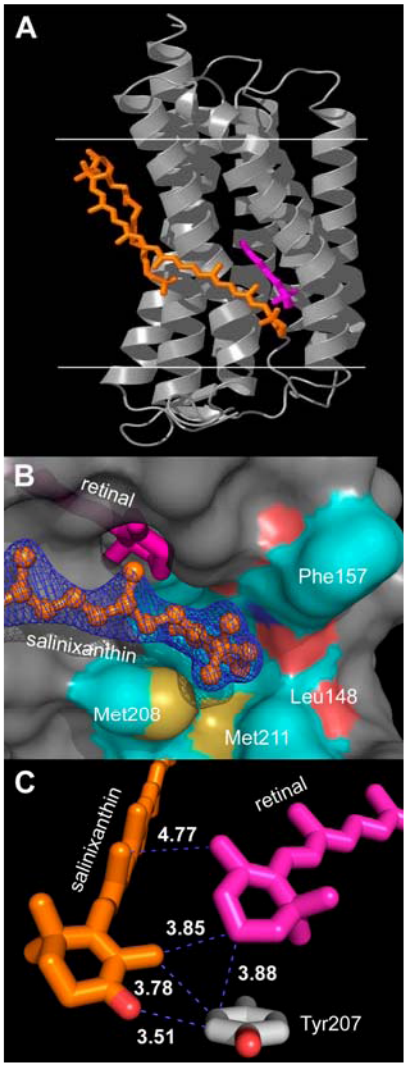

Figure 8. Location of salinixanthin (orange) and retinal (magenta) in xanthorhodopsin. (A) The extended carotenoid is tightly bound on the surface of xanthorhodopsin. Horizontal lines indicate the approximate boundaries of the lipid bilayer. (B) The binding pocket of the salinixanthin keto ring is formed by Leu[148], Gly[156], Phe[157], Thr[160], Met[208], and Met[211], as well as the retinal β-ionone ring. (C) The keto ring of the carotene is rotated 82° out of plane of the salinixanthin-conjugated system and is in van der Waals distance of the retinal β-ionone and the phenolic side chain of Tyr[207]. From **[4].**

b) Carotenoid conformation and residues that constitute its binding site. The carotenoid lies transverse against the outer surface of helix F at a 54° angle to the membrane normal, buried at the protein-lipid boundary. Its keto ring is immobilized by residues at the extracellular ends of helices E and F and by the β-ionone ring of the retinal, and rotated 82° from the plane of the methyl group of its polyene chain and therefore from the plane of the extended π–system. The keto group oxygen is not hydrogen-bonded. The immobilized and acutely out-of-plane orientation of the keto ring minimizes participation of its two double bonds in the conjugated π-system, and explains the well-resolved vibronic bands of the carotenoid, the lack of a red-shift of the bands upon binding, and the strong CD bands in the visible region. The relatively rigid polyene is wedged in a slot on the outside of helix F, with one side formed by the Leu[194] and Leu[197] side-chains and the other by the Ile[205] side-chain. The carotenoid glucoside is hydrogen-bonded to the C=O and the NH_2 of the amide side-chain of Asn[191], as well as NH1 of Arg[184]. The dependence of the carotenoid spectrum on the retinal is explained by the fact that the retinal β-ionone ring is part of the carotenoid binding site.

The keto ring of the carotenoid is in the space occupied by Trp[138] in bacteriorhodopsin, one of the bulky side-chains that stabilizes the retinal ionone ring in that protein [20]. In xanthorhodopsin it is replaced by a glycine. Another difference is Glu[141] of xanthorhodopsin, which is alanine in bacteriorhodopsin but a conserved glutamate in proteorhodopsins involved in spectral tuning.

c) Large differences in structure compared to bacteriorhodopsin. Remarkably, there are greater differences from the main-chain of bacteriorhodopsin than in any of the previously crystallized microbial rhodopsins. Helices A and G are longer by four and nine residues, respectively, and their tilt and rotation, particularly of helix A, are considerably different. In bacteriorhodopsin the interhelical B-C antiparallel β-sheet interacts with the D-E loop, while in xanthorhodopsin it reorients dramatically to interact with the Arg[8] peptide C=O near the N-terminus, where it forms a mini three-stranded β-sheet. As a result, the tip of the B-C loop is displaced, by 30 Å, toward the periphery of the protein. Unexpected in a heptahelical membrane protein, a large cleft is formed that extends far into the inte-

rior and brings the aqueous interface near functional residues that are buried in other rhodopsins. This feature correlates with a different mechanism of proton release in xanthorhodopsin (see below).

d) The hydrogen bonding network in the extracellular channel in xanthorhodopsin is very different from that in bacteriorhodopsin. In bacteriorhodopsin, Wat^{402} receives a hydrogen bond from the protonated retinal Schiff base and donates hydrogen bonds to the two anionic residues, Asp^{85} and Asp^{212} [20] and this arrangement is conserved in xanthorhodopsin. However, in xanthorhodopsin the carboxylate of Asp^{96} (homologous to Asp^{85} in bacteriorhodopsin) is severely rotated, and the hydrogen-bonded aqueous network of water molecules in the extracellular region that facilitates proton release in the photocycle is replaced by hydrogen-bonded residues that are likely to be more resistant to rearrangement than an aqueous network. In bacteriorhodopsin, a pair of glutamate residues, Glu^{194} and Glu^{204}, stabilizes a hydrogen-bonded water, the source of the proton released to the extracellular surface after the retinal Schiff base is deprotonated [21]. The eubacterial pumps, proteorhodopsin and xanthorhodopsin, contain only one of these acidic groups. Further, in xanthorhodopsin, at least, the single glutamate is far removed from Arg^{93} (> 18 Å vs. 7.3 Å in bacteriorhodopsin). In bacteriorhodopsin, release of the proton is triggered by the movement of the positively charged Arg^{82} side-chain toward the glutamate pair, upon protonation of Asp^{85} [22]. This is unlikely to occur in the xanthorhodopsin photocycle, because NH1 and NH2 of Arg^{93} are both hydrogen-bonded to the peptide carbonyl of Gln^{229} instead of water molecules.

e) Asp-His hydrogen bonded pair is the counterion in xanthorhodopsin. One of the distinguishing features of eubacterial proton pumps is that the pK_a of the primary proton acceptor is not as low as 2.5 in bacteriorhodopsin, but near 7 [14, 15]. The origin of the increased proton affinity, which makes these proteins functional as pumps only at alkaline pH, has been an unsolved problem. In xanthorhodopsin, ND1 of His^{62} is hydrogen-bonded to OD1 of Asp^{96} (**Figure 9**). At 2.4-2.5 Å distance, this is a very short hydrogen-bond. Thus, its proton may be shared by the imidazole ring and the carboxylate in a single-well, strong hydrogen bond. This complex, with an expected pK_a higher than the aspartate alone, must be regarded as the Schiff base counterion. If analogy with bacteriorhodopsin holds, the anionic, rather than the neutral complex, is the proton acceptor of the Schiff base in the photocycle.

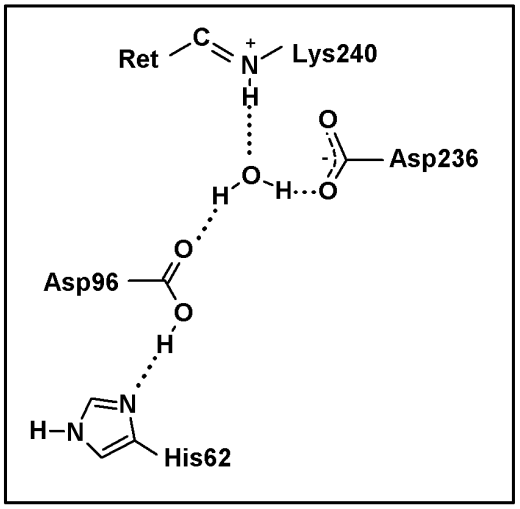

Figure 9. Asp-His counterion to the Schiff base of xanthorhodopsin. The Schiff base interacts with the counterion through a water molecule ($Water^{402}$). After [4].

A histidine at this position (see **Figure 7**) is highly conserved in the proteorhodopsins, making it likely that the aspartate-histidine complex is a general characteristic of eubacterial pumps. Once protonated in the photocycle, the His^{62}-Asp^{96} complex would be a good candidate for the origin of the proton released to the medium upon deprotonation of the retinal Schiff base, but at neutral pH, at least, such early proton release does not occur; it is released at the end of photocycle. Asp-His pair connected with a strong short hydrogen bond is involved in catalysis of different reactions in many enzymes such as α-chymotrypsin [23].

f) Pre-arrangement of the hydrogen bonding network in the cytoplasmic channel. In the cytoplasmic region of bacteriorhodopsin, the proton donor is in an anhydrous environment that constitutes the hydrophobic barrier in the cytoplasmic half of the protein [24, 25]. This, and the fact that it donates a hydrogen-bond to OD1 of Thr^{46}, raises its pK_a. The aspartic acid becomes a proton donor to the Schiff base during the photocycle only after hydration of this region that includes a hydrogen-bonded chain of four water molecules to connect the proton donor to its acceptor [26]. In xanthorhodopsin, as in the proteorhodopsins, these residues are replaced by a glutamic acid and a serine. The carboxyl is hydrogen-bonded to Wat^{502} that connects to the peptide carbonyl of Lys^{240}. It appears therefore, that in xanthorhodopsin part of the cytoplasmic hydrogen-bonded chain of water molecules between the retinal and the proton donor is in position favorable for proton transport already in the initial state, which might explain accelerated reprotonation of the Schiff base in the photocycle.

10. Femtosecond dynamics of salinixanthin and retinal excited states and energy transfer [27]. The strong absorption of carotenoids in the blue-green spectral region is caused by a transition to the excited state called S_2 ($1B_u^+$ in the C_{2h} symmetry group notation). For symmetry reasons, the transition to the lowest excited state S_1, ($1A_g^-$) from the ground state is forbidden, but the S_1 state is populated via S_2-to-S_1 internal conversion on a time scale of few hundred femtoseconds [17]. Both S_2 and S_1 states were identified as energy donors in carotenoid-(bacterio)chlorophylls antenna systems. For carotenoids with conjugation length N > 11 intermediate states could present between the S_2 and S_1 states, making them all potential energy donors in the energy transfer process.

In xanthorhodopsin, the lowest excited state of the retinal chromophore is too high to allow for energy transfer from the S_1 state of salinixanthin. Consequently, salinixanthin-to-retinal energy transfer was suggested to occur exclusively from the salinixanthin S_2 state [1]. Strong evidence for that was obtained from fluorescence excitation spectra and steady state carotenoid fluorescence changes upon the Schiff base reduction with borohydride [11]. Nevertheless direct time-resolved methods are necessary to determine the energy transfer rates, dynamics of the excited states and verify the energy transfer pathway(s).

Using femtosecond pump-probe set-up with a time resolution of 40 fs and measuring kinetics of light-induced absorption changes at 900 nm, it was possible to follow the decay of the S_2 excited state of salinixanthin. The 900 nm was chosen as a probing wavelength, because at this wavelength the carotenoids usually exhibit strong S_2-S_n transition [28]. Simultaneously, one can monitor the appearance of the product of energy transfer, i.e., the excited retinal chromophore, that has a stimulated emission in the 800-950 nm region [29]. The obtained kinetics are shown in **Figure 10**.

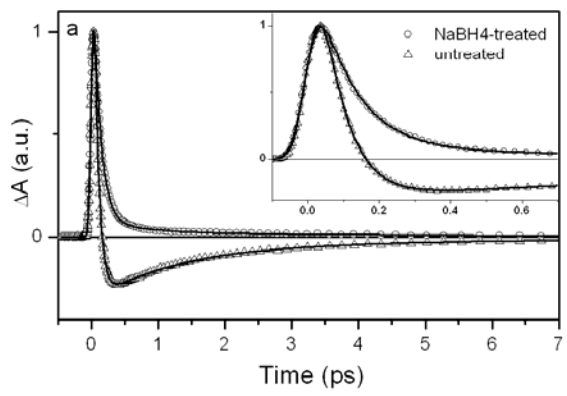

Figure 10. Kinetics recorded at the maximum of stimulated emission of retinal chromophore, 900 nm, after excitation at 490 nm. Solid lines represent fits.

The instantaneous positive signal is due to the S_2-S_n transition. The initial decay in the sample treated with sodium borohydride (to eliminate energy transfer by causing a large blue

shift in the retinal chromophore band) can be fitted with a 110 fs time constant which characterizes the intrinsic S_2 lifetime of salinixanthin in xanthorhodopsin. The kinetic trace obtained after 490-nm excitation of untreated (native) xanthorhodopsin clearly demonstrates energy transfer from salinixanthin to retinal. The decay of the S_2-S_n signal of salinixanthin produces a negative signal that is due to stimulated emission of the excited retinal. Based on the analysis of the data we conclude that the S_2 lifetime of the bound salinixianthin is in the 66 ± 4 fs range. The difference in the life times in treated and untreated xanthorhodopsin corresponds to 40 ± 4% energy transfer, in agreement with estimates from excitation spectra [11]. The stimulated emission of retinal decays with a time constant of 1.2 ps, significantly slower than the 0.7 ps observed for bacteriorhodopsin [29, 30]. Preliminary experiments on femtosecond up-conversion fluorescence kinetics indicate that even longer components in the retinal excited states lifetime are present.

Publications credited to the ARO grant

With the support of the ARO Grant W911NF-06-1-0020 (starting date December 1, 2005, end date February 28, 2009) the following papers have been published in peer reviewed journals:

Balashov, S. P., E. S. Imasheva and J. K. Lanyi. 2006. Induced chirality of light-harvesting carotenoid salinixanthin and its interaction with the retinal of xanthorhodopsin. *Biochemistry* **45**, 10998-11004.

Imasheva, E. S., S. P. Balashov, J. M. Wang and J. K. Lanyi. 2006. pH-dependent transitions in xanthorhodopsin. *Photochem. Photobiol.* **82**, 1406-1413.

Boichenko, V. A., J. M. Wang, J. Antón, J. K. Lanyi and S. P. Balashov. 2006. Functions of carotenoids in xanthorhodopsin and archaerhodopsin, from action spectra of photoinhibition of cell respiration. *Biochim. Biophys. Acta* **1757**, 1649-1656.

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Polívka, T., S. P. Balashov, P. Chábera, E. S. Imasheva, A. Yartsev, V. Sundström and J. K. Lanyi. 2009. Femtosecond Carotenoid to Retinal Energy Transfer in Xanthorhodopsin. *Biophys. J.* 96, 2268-2277.

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